Appendix B

continue past the stopping plate, penetrating the cells. When such a system was used for transfecting maize cells with an expressable chloramphenicol acetyltransferase (CAT) gene, CAT activity up to 200-fold over background was observed 24–96 hr after bombardment. Repeated bombardments of the same cell samples resulted in a proportionate increase in the observed levels of CAT activity. The major advantages of this system for gene transfer into maize cells are elimination of the need to generate protoplasts prior to transfection and elimination of the subsequent difficulty of generating whole plants from transfected protoplasts.

The applicability of this method to mammalian cells has not been determined. Factors believed to be important to the success of the method include the size of the microprojectile, the velocity at which the microprojectiles are delivered, and the atmospheric pressure under which the bombardment takes place.

[42] Selection and Coamplification of Heterologous Genes in Mammalian Cells

By RANDAL J. KAUFMAN

Introduction

In the early 1950s, investigators established cell culture systems to study the mechanism by which cancer cells become resistant to a variety of chemotherapeutic agents, such as methotrexate (MTX). The initial investigations led to the observation that stepwise selection for growth of cultured animal cells in progressively increasing concentrations of MTX results in cells with increased levels of the target enzyme, dihydrofolate reductase (DHFR), as a consequence of a proportional increase in the DHFR gene copy number.[1] Subsequently, it has been observed that gene amplification is ubiquitous in nature and many, if not all, genes become amplified at some frequency (approximately $1/10^4$, although this number can vary extensively) (for recent review, see Ref. 2). With appropriate selection conditions, where the growth of cells harboring amplification of a particular gene is favored, a population of cells that contain the amplified gene will outgrow the general population. In the absence of drug selection, the amplified gene is most frequently lost.

Since the degree of gene amplification, in most cases, is proportional to

[1] F. W. Alt, R. E. Kellems, J. R. Bertino, and R. T. Schimke, *J. Biol. Chem.* **253**, 1357 (1978).
[2] R. T. Schimke, *J. Biol. Chem.* **263**, 5989 (1988).

the level of gene expression, it offers a convenient means to increase expression of any particular gene. Although the copy number of a wide variety of genes can be amplified as a consequence of applying appropriate selective pressures, for many genes, direct selection methods are not available. In these cases, it is possible to introduce the gene of interest with a selectable and amplifiable marker gene into the cell and subsequently select for amplification of the marker gene to generate cells that have coamplified the desired gene. DHFR is the most widely used amplifiable marker gene for this purpose. This article reviews the characteristics of gene amplification and describes the methods and available selectable genetic markers for cotransfection and coamplification to obtain high-level expression of heterologous genes in mammalian cells.

Characteristics of Gene Amplification

Several general features are characteristic of gene amplification in cultured mammalian cells: (1) amplification is usually obtained after stepwise selection for increasing resistance, (2) the amplified DNA displays variable stability, and (3) the size and structure of the amplified unit are variable and these characteristics may change with time. These characteristics suggest that multiple mechanisms are likely responsible for gene amplification.

Stepwise Selection

Gene amplification generally occurs as a result of stepwise selection for resistance to increasing concentrations of the selective agent. When larger selection steps are employed, mechanisms other than gene amplification are observed. For example, single-step high-level resistance to MTX may result in cells with an altered, MTX-resistant DHFR enzyme[3,4] or in cells that have altered MTX transport properties.[5,6] Since gene amplification requires DNA replication and cell division, optimal amplification occurs when cells are subject to severe, but not absolute growth-limiting conditions. In some cases, resistant colonies may appear but fail to continue growing, whereas in other cases, only after prolonged selection does the growth rate eventually return to that of the original parental line. These observations suggest that many events may occur from the time a cell first becomes resistant until it is established as a drug-resistant clonal line.

[3] W. F. Flintoff, S. V. Davidson, and L. Siminovitch, *Somatic Cell Genet.* **2,** 245 (1976).
[4] D. A. Haber, S. M. Beverley, and R. T. Schimke, *J. Biol. Chem.* **256,** 9501 (1981).
[5] F. M. Sirotnik, S. Kurita, and D. J. Hutchison, *Cancer Res.* **28,** 75 (1968).
[6] Y. Assaraf and R. T. Schimke, *Proc. Natl. Acad. Sci. U.S.A.* **84,** 7154 (1987).

A number of approaches have been taken to increase the frequency of gene amplification. Perturbation of DNA synthesis by treatment with hydroxyurea,[7] aphidicolin,[7] UV γ irradiation,[8] hypoxia,[9] carcinogens,[10] and arsenate[11] can enhance the frequency of specific gene amplification 10- to 100-fold. Agents which promote cell growth, such as phorbol esters or insulin, can also increase the frequency of gene amplification.[12] Interestingly, cells resistant to one selective agent as a result of gene amplification exhibit increased frequencies for amplification of other genes.[13] This "amplificator" phenotype may be one particular property of cells selected for stepwise increasing levels of drug resistance or it may reflect the finding that disruption of DNA replication results in an enhancement of gene amplification events in general.[14]

Variable Stability of Amplified Genes

When drug-resistant cells are propagated in the absence of the selective agent, the amplified genes may be maintained or lost. The amplified genes in newly selected resistant cell lines are unstable. As cells are propagated for increasing periods of time in the presence of the selection agent, the amplified genes exhibit increased stability.[15,16] The degree of stability of the amplified genes in the absence of drug selection may correlate with the localization of the amplified genes and the complexity of the associated karyotypic alterations. Amplified genes localized to double minute chromosomes are usually lost upon propagation in the absence of selection.[16] Double minute chromosomes are small paired extrachromosomal elements which lack centromeric function and thus, segregate randomly at mitosis. Most evidence suggests that double minute chromosomes are circular DNA structures.[17] In contrast, stably amplified genes are usually integrated into the chromosome and frequently are associated with expanded chromosomal regions termed homogeneously staining regions

[7] C. A. Hoy, G. C. Rice, M. Kovacs, and R. T. Schimke, *J. Biol. Chem.* **262,** 11927 (1987).
[8] T. D. Tlsty, P. C. Brown, and R. T. Schimke, *Mol. Cell. Biol.* **4,** 1050 (1984).
[9] G. C. Rice, C. Hoy, and R. T. Schimke, *Proc. Natl. Acad. Sci. U.S.A.* **83,** 5978 (1986).
[10] S. Lavi, *Proc. Natl. Acad. Aci. U.S.A.* **78,** 6144 (1981).
[11] T. C. Lee, P. W. Lamb, N. Tanaka, T. N. Gilmer, and J. C. Barrett, *Science* **241,** 79 (1988).
[12] A. Varshavsky, *Cell* **25,** 561 (1981).
[13] E. Giulotto, C. Knights, and G. R. Stark, *Cell* **48,** 837 (1987).
[14] R. N. Johnston, J. Feder, A. B. Hill, S. W. Sherwood, and R. T. Schimke, *Mol. Cell. Biol.* **6,** 3373 (1986).
[15] R. J. Kaufman and R. T. Schimke, *Mol. Cell. Biol.* **1,** 1069 (1981).
[16] R. J. Kaufman, P. C. Brown, and R. T. Schimke, *Proc. Natl. Acad. Sci. U.S.A.* **76,** 5669 (1979).
[17] B. Hamkalo, P. J. Farnham, R. Johnston, and R. T. Schimke, *Proc. Natl. Acad. Sci. U.S.A.* **82,** 1126 (1985).

(HSRs).[18] This term reflects the lack of banding in these regions after Giemsa–trypsin banding procedures. Both amplified endogenous[19] and heterologous[20] DHFR genes in MTX-resistant mouse fibroblasts are usually associated with double minute chromosomes. In contrast, amplified endogenous and transfected heterologous DHFR genes in MTX-resistant Chinese hamster ovary (CHO) cells are frequently associated with expanded chromosomal regions.[18,21] In a few cases, the amplified transfected DNA may be present with little cytological perturbation.[21] This may reflect a smaller size of the amplified unit within the chromosome. CHO cells selected in the presence of MTX for amplification of heterologous DHFR genes have generally proved to be very stable upon propagation in the absence of MTX.[18] Instability associated with chromosomal amplified genes may correlate with more complex chromosomal alterations. For example, cells selected for amplified genes may become tetraploid, a characteristic possibly associated with increased instability. In other cases, transfected and amplified DNA may be observed in HSRs associated with extremely large or dicentric chromosomes. These chromosomes are prone to breakage events which may be responsible for instability.[21–24]

Variable Size and Structure of Amplified Unit

The size of the amplified unit estimated by cytogenetic analysis is highly variable and may range from less than 100 kilobase pairs to greater than 500 kilobase pairs.[18,25] In several cases, the amplified DNA consists of inverted duplications.[25–27] During propagation of cells in culture, the amplified DNA may undergo changes which involve (1) extrachromosomal circles becoming larger through tandem duplications,[28,29] (2) the positions

[18] J. H. Nunberg, R. J. Kaufman, R. T. Schimke, G. Urlaub, and L. A. Chasin, *Proc. Natl. Acad. Sci. U.S.A.* **75**, 5553 (1978).
[19] P. C. Brown, S. M. Beverly, and R. T. Schimke, *Mol. Cell. Biol.* **1**, 1077 (1981).
[20] M. J. Murray, R. J. Kaufman, S. A. Latt, and R. A. Weinberg, *Mol. Cell. Biol.* **3**, 32 (1983).
[21] R. J. Kaufman, P. A. Sharp, and S. A. Latt, *Mol. Cell. Biol.* **3**, 699 (1983).
[22] D. M. Robins, R. Axel, and A. S. Henderson, *J. Mol. Appl. Genet.* **1**, 191 (1981).
[23] B. Fendrock, M. Destremps, R. J. Kaufman, and S. A. Latt, *Histochemistry* **84**, 121 (1986).
[24] J. L. Andrulis and L. Siminovits, *in* "Gene Amplification" p. 75. (R. T. Schimke, ed.). Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1982.
[25] J. E. Looney and J. L. Hamlin, *Mol. Cell. Biol.* **7**, 569 (1987).
[26] M. Ford and M. Fried, *Cell* **45**, 425 (1986).
[27] J. C. Ruiz and G. M. Wahl, *Mol. Cell. Biol.* **8**, 4302 (1988).
[28] S. M. Carroll, M. L. DeRose, P. Gaudray, and C. M. Moor, *Mol. Cell. Biol.* **8**, 1525 (1988).
[29] N. A. Federspeil, S. M. Beverley, J. W. Schilling, and R. T. Schimke, *J. Biol. Chem.* **259**, 9127 (1984).

of novel recombination breakpoints between amplified units may change,[29,30] or (3) the propagation of mutations from one amplified unit to other units within an amplified array, possibly by gene conversion.[31]

The mechanism of amplification of a transfected CAD gene upon selection for PALA resistance has been extensively studied.[27,28,32] The integrated CAD sequence is first deleted and then undergoes replication as an extrachromosomal element. The extrachromosomal intermediate may increase in size by forming multiples of itself to eventually create double minute chromosomes. The extrachromosomal sequence may eventually integrate to form a homogeneously staining chromosomal region.

Amplification of Cotransfected DNA

With most methods of DNA transfer, 5–50% of the cells in the population acquire DNA and express it transiently for several days to several weeks. However, the DNA is eventually lost from the cell population unless some selection procedure is used to iosolate cells that have stably integrated the foreign DNA into their genome. The limiting event for obtaining stable DNA transfer is the frequency of DNA integration and not the frequency of DNA uptake into the cell. Different cell lines as well as different transfection methods yield dramatically different efficiencies with respect to the frequency of stable integration, as well as to the amount of foreign DNA incorporated. The ability to select for incorporation of one gene by selection for a second cotransfected gene has been termed cotransformation.[33] In cotransformation, separate DNA molecules become ligated together inside the cell and subsequently cointegrate as a unit via nonhomologous recombination into the host chromosome. When separate DNA molecules are sequentially introduced into recipient cells, the molecules do not become linked and are not cointegrated into the same chromosomal position. If DNA molecules are linked together within the chromosome, selection for amplification of one of the molecules usually results in coamplification of the linked DNA molecule. Different cell lines and DNA transfection methods exhibit different potentials for cotransformation. For example, the frequency of cotransformation in CHO cells is lower than that observed in mouse L cells. In this case, the difference may be attributa-

[30] E. Giulotto, I. Saito, and G. R. Stark, *EMBO J.* **5**, 2115 (1986).
[31] J. M. Roberts and R. Axel, *Cell* **29**, 109 (1982).
[32] S. Carroll, P. Gaudray, M. DeRose, J. Emery, J. Meinkoth, E. Nakkim, M. Subler, D. Von Hoff, and G. Whal, *Mol. Cell. Biol.* **7**, 1740 (1987).
[33] M. Wigler, S. Silverstein, L. S. Lee, A. Pellicer, Y. Cheng, and R. Axel, *Cell* **11**, 223 (1977).

ble to the lesser amount of DNA incorporated into CHO cells compared to mouse L cells. Cotransformation by $CaPO_4$ DNA-mediated transfection is very efficient,[33] whereas cotransformation by protoplast fusion of separate bacteria harboring two independent plasmids is very rare. In situations where cotransformation is inefficient, it is better to use plasmid vectors which express both the product gene and the selection gene. Vectors useful for this purpose are described below and in Fig. 3.

In order to coamplify a particular DNA sequence, it is important that the DNA sequence or its products not interfere with amplification or be toxic to the cell. Since a minor subset of cells from the total population survives during each step of the selection process, it is possible to select for mutations in the coamplified gene which permit cell viability. Thus, the protein obtained after selection for expression at high level may be different from that encoded by the original transfected gene. For example, selection for coamplification of DHFR and SV40 small t antigen in CHO cells resulted in highly MTX-resistant cells which expressed SV40 small t antigen at 15% of the total protein synthesis.[34] However, the t antigen expressed at high level migrated at a molecular weight of 2000 less than the wild-type t antigen from the same cells at a lower expression level. Although the basis for this change was not characterized, it demonstrates the potential difficulty in assuring the fidelity of proteins produced after selection for gene amplification.

Amplifiable Genetic Markers

Resistance to cytotoxic drugs is the characteristic most widely used to select for stable transformants. Drug resistance can be recessive or dominant. Genes conferring recessive drug resistance require a particular host which is deficient in the activity which is being selected. Genes conferring dominant drug resistance can be used independent of the host. Many of the recessive genetic selectable markers are involved in the salvage pathway for purine and pyrimidine biosynthesis. When *de novo* biosynthesis of purines or pyrimidines is inhibited, the cell can utilize purine and pyrimidine salvage pathways, providing that the enzymes (i.e., thymidine kinase, hypoxanthine-guanine phosphoribosyltransferase, adenine phosphoribosyltransferase, or adenosine kinase) necessary for conversion of the nucleoside precursors to the corresponding nucleotides are present (see Fig. 1). Since these salvage enzymes are not required for cell growth when *de novo* purine or pyrimidine biosynthesis is functional, cells deficient for a particular salvage pathway enzyme are viable under normal growth conditions. How-

[34] R. J. Kaufman and P. A. Sharp, *J. Mol. Biol.* **159**, 601 (1982).

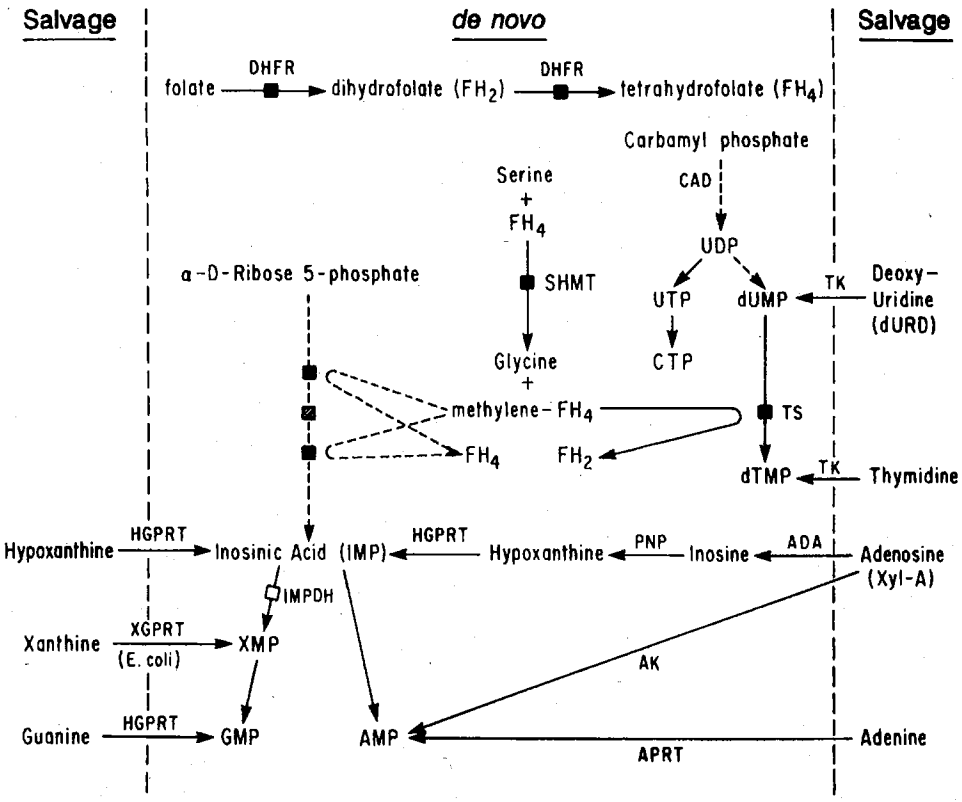

FIG. 1. *De novo* and salvage biosynthetic pathways for purines and pyrimidines involving available selectable markers. *De novo* enzymes: DHFR, dihydrofolate reductase; CAD, carbamoyl-P synthase, aspartate transcarbamoylase, and dihydroorotase (only the latter two activities are indicated); SHMT, serine hydroxymethyltransferase; TS, thymidylate synthase; IMPDH, inosine-monophosphate dehydrogenase. Salvage enzymes: TK, thymidine kinase; ADA, adenosine deaminase; PNP, purine nucleoside phosphorylase; AK, adenosine kinase; APRT, adenine phosphoribosyltransferase; HGPRT, hypoxanthine-guanine phosphoribosyltransferase; XGPRT, *E. coli* xanthine-guanine phosphoribosyltransferase. Solid arrows indicate single reactions. Dashed arrows indicate multiple reactions. Solid squares indicate reactions that are inhibited by the folate analogs methotrexate and aminopterin. The hatched square indicates the principal reaction inhibited by azaserine. The open square indicates the reaction inhibited by mycophenolic acid. [Reprinted with permission from R. J. Kaufman, *in* "Genetic Engineering" (J. K. Setlow, ed.), Vol. 9, p. 155. Plenum, New York, 1988.]

ever, addition of drugs which inhibit the *de novo* biosynthesis of purines or pyrimidines results in death of deficient cells because the salvage pathway becomes essential to provide purines and pyrimidines. Cells which acquire the capability to express the deficient activity via gene transfer can be selected for growth under these conditions. This has become a common

selection technique and is the basis for the thymidine kinase (TK), adenine phosphoribosyltransferase (APRT), and hypoxanthine-guanine phosphoribosyltransferase (HGPRT) selection.

Many selectable recessive genetic markers can be used in a dominant fashion if there is a suitable drug to titrate the activity of the marker gene product and if there is a suitable expression vector to produce significantly more gene product than is present in the host cell to be transfected. Table I lists genes for which selection schemes for gene amplification are available. Most, if not all, the genes listed in Table I could be used as selectable markers for coamplification. In addition to these genes, a large number of genes can become amplified upon transformation and tumor progression.[35] However, there is no direct means for which to select for their amplification. The most useful dominant and amplifiable genetic marker selection systems for coamplification of heterologous genes are described below.

Dihydrofolate Reductase–Methotrexate Resistance

Dihydrofolate reductase (DHFR) catalyzes the conversion of folate to tetrahydrofolate (FH4). FH4 is required for the biosynthesis of glycine from serine, for the biosynthesis of thymidine monophosphate from deoxyuridine monophosphate, and for purine biosynthesis (see Fig. 1). MTX is a folic acid analog which binds and inhibits DHFR, leading to cell death. When cells are selected for growth in sequentially increasing concentrations of MTX, the surviving population contains increased levels of DHFR which result from an amplification of the DHFR gene.[1,2] Highly MTX-resistant cells may contain several thousand copies of the DHFR gene and express several thousandfold elevated levels of DHFR. MTX resistance selection for DHFR gene amplification is the most convenient and widely used system for coamplification to obtain high-level expression of heterologous genes.

The convenience of the DHFR selection system relies on the availability of CHO cells that are deficient in DHFR.[36] The origin and genetics of CHO cells have been reviewed.[37] DHFR-deficient CHO cells were isolated after ethylmethane sulfonate and UV γ irradiation induced mutagenesis and selection for growth in high-specific-activity [^{3}H]deoxyuridine.[36] Cells containing functional DHFR convert [^{3}H]deoxyuridine to [^{3}H]thymidylate, which is incorporated into DNA. This incorporation into DNA is lethal due to disruption of the DNA by radioactive damage. DHFR-deficient cells survive this selection procedure. The DHFR-deficient cells re-

[35] K. Alitalok, *Med. Biol.* **62**, 304 (1984).
[36] G. Urlaub and L. A. Chasin, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 4216 (1980).
[37] M. M. Gottesman (ed.), "Molecular Cell Genetics." Wiley, New York, 1985.

TABLE I
GENE AMPLIFICATION IN DRUG-RESISTANT MAMMALIAN CELLS

Selection	Gene	Ref.[a]
Methotrexate	Dihydrofolate reductase	(*1*)
Cadmium	Metallothionein	(*2*)
PALA	CAD	(*3*)
Xyl-A-or adenosine and 2′-deoxycoformycin	Adenosine deaminase	(*4*)
Adenine, azaserine, and coformycin	Adenylate deaminase	(*5*)
6-Azauridine, pyrazofuran	UMP Synthetase	(*6*)
Mycophenolic acid	IMP 5′-dehydrogenase	(*7*)
Mycophenolic acid with limiting xanthine	Xanthine-guanine phosphoribosyltransferase	(*8*)
Hypoxanthine, aminopterin, and thymidine (HAT)	Mutant HGPRTase or mutant thymidine kinase	(*9*, *10*)
5-Fluorodeoxyuridine	Thymidylate synthetase	(*11*)
Multiple drugs[b]	P-glycoprotein 170	(*12*)
Aphidicolin[c]	Ribonucleotide reductase	(*13*)
Methionine sulfoximine	Glutamine synthetase	(*14*)
β-Aspartyl hydroxamate or Albizziin	Asparagine synthetase	(*15*)
Canavanine	Arginosuccinate synthetase	(*16*)
α-Difluoromethylornithine	Ornithine decarboxylase	(*17*)
Compactin	HMG-CoA reductase	(*18*)
Tunicamycin[c]	*N*-Acetylglucosaminyl transferase	(*19*)
Borrelidin[c]	Threonyl-tRNA synthetase	(*20*)
Ouabain	Na^+, K^+-ATPase	(*21*)

[a] Key to references: (*1*) F. W. Alt, R. E. Kellems, J. R. Bertino, and R. T. Schimke, *J. Biol. Chem.* **253**, 1357 (1978); (*2*) L. R. Beach and R. D. Palmiter, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 210 (1981); (*3*) G. M. Wahl, R. A. Padgett, and G. R. Stark, *J. Biol. Chem.* **254**, 8679 (1979); (*4*) C. Y. Yeung, D. E. Ingolia, C. Bobonis, B. S. Dunbar, M. E. Riser, J. J. Siciliano, and R. E. Kellems, *J. Biol. Chem.* **258**, 8338 (1983); (*5*) M. Debatisse, O. Hyrien, E. Petit-Koskas, B. R. de Saint-Vincent, and G. Buttin, *Mol. Cell. Biol.* **6**, 1776 (1986); (*6*) J. J. Kanalas and D. P. Suttle, *J. Biol. Chem.* **259**, 1848 (1984); (*7*) E. Huberman, C. K. McKeown, and J. Friedman, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 3151 (1981); (*8*) A. B. Chapman, M. A. Costello, R. Lee, and G. M. Ringold, *Mol. Cell. Biol.* **3**, 1421 (1983); (*9*) J. Brennand, A. C. Chinault, D. S. Konecki, D. W. Melton, and C. T. Caskey, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 1950 (1982); (*10*) J. M. Roberts and R. Axel, *Cell* **29**, 109 (1982); (*11*) C. Rossana, L. G. Rao, and L. F. Johnson, *Mol. Cell. Biol.* **2**, 1118 (1982); (*12*) J.R. Riordan, K. Deuchars, N. Kartner, N. Alon, J. Trent, and V. Ling, *Nature (London)* **316**, 817 (1985); (*13*) C. L. K. Sabourin, P. F. Bates, L. Glatzer, C.-C. Chang, J. E. Trosko, and J. A. Boezi, *Somatic Cell Genet.* **7**, 255 (1981); (*14*) A. P. Young and G. M. Ringold, *J. Biol. Chem.* **258**, 11260 (1983); (*15*) I. L. Andrulis, C. Duff, S. Evans-Blackler, R. Worton, and L. Siminovitch, *Mol. Cell. Biol.* **3**, 391 (1983); (*16*) T. S. Su, G. G. O. Bock, W. E. O'Brein, and A. L. Beaudet, *J. Biol. Chem.* **256**, 11826 (1981); (*17*) L. McConlogue and P. Coffino, *J. Biol. Chem.* **258**, 12083 (1983); (*18*) K. L. Luskey, J. R. Faust, D. J. Chin, M. S. Brown, and J. L. Goldstein, *J. Biol. Chem.* **258**, 8462 (1983); (*19*) B. A. Crisculolo and S. S. Krag, *J. Cell Biol.* **94**, 586 (1982); (*20*) J. S. Gantt, C. A. Bennett, and S. M. Arfin, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 5367 (1981); (*21*) P. G. Pauw, M. D. Johnson, P. Moore, M. Morgan, R. M. Fineman, T. Kalka, and J. F. Ash, *Mol. Cell. Biol.* **6**, 1164 (1986).

[b] Adriamycin, vincristine, colchicine, actinomycin D, puromycin, cytocholasin B, emetine, maytansine, Bakers' antifolate.

[c] Examples of increased enzyme activity likely resulting from gene amplification.

quire the addition of thymidine, glycine, and hypoxanthine to the growth medium, and do not grow in the absence of added nucleosides unless they acquire a functional DHFR gene. Most frequently used cell clones for heterologous expression are DUKX-B11 (DXB-11) (isolated from the proline auxotroph CHO-K1) and DG44 [isolated from the CHO (Toronto) cell line]. These cell lines require the presence of a purine source (either hypoxanthine or adenosine and deoxyadenosine), thymidine, and glycine to complement the DHFR deficiency. These lines can be obtained from Dr. Lawrence Chasin, Columbia University, NY.

Coamplification of heterologous genes with DHFR in DHFR-deficient CHO cells can yield cell lines that express high levels of a protein from heterologous genes. The advantages of CHO cells for the expression of heterologous genes include (1) the amplified genes are integrated into the host chromosome and as a result are stably maintained even in the absence of continued drug selection, (2) a variety of proteins have been properly expressed and secreted at high levels in CHO cells, (3) CHO cells adapt well to growth in the absence of serum, (4) CHO cells can grow either attached or in suspension, and (5) CHO cells have been scaled up to greater than 5000 liters. Among the genes expressed in this manner are human tissue plasminogen activator,[38] human interferon γ,[39,40] human interferon β,[41] human factor IX,[42] human factor VIII,[43] the herpes simplex glycoprotein D,[44] and the α and β subunits of bovine luteinizing hormone.[45]

Transformant Selection and Coamplification of DHFR in DHFR-Deficient CHO Cells. GROWTH OF DHFR-DEFICIENT CHO CELLS. Medium: Ham's nutrient mixture F12 was specially designed for growth of CHO cells. Alternatively, alpha medium may be used in place of Ham's F12. Both media can be ordered specifically without nucleosides from Gibco Inc. (Grand Island, NY). For complementation of the DHFR deficiency, the following supplements need to be added if they are not present in the growth medium: hypoxanthine (10 μg/ml) or adenosine (10 μg/ml) and deoxyadenosine (10 μg/ml), thymidine (10 μg/ml), glycine (50 μg/ml). In

[38] R. J. Kaufman, L. C. Wasley, A. T. Spiliotes, S. D. Gossels, S. A. Latt, G. R. Larsen, and R. M. Kay, *Mol. Cell. Biol.* **5**, 1730 (1985).
[39] J. Haynes and C. Weissman, *Nucleic Acids Res.* **11**, 687 (1983).
[40] S. J. Schahill, R. Devos, J. V. Heyden, and W. Fiers, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 4654 (1983).
[41] F. McCormack, M. Trahey, M. Innis, B. Dieckmann, and G. Ringold, *Mol. Cell. Biol.* **4**, 166 (1984).
[42] R. J. Kaufman, L. C. Wasley, B. C. Furie, B. Furie, and C. Shoemaker, *J. Biol. Chem.* **261**, 9622 (1986).
[43] R. J. Kaufman, L. C. Wasley, and A. J. Dorner, *J. Biol. Chem.* **263**, 6362 (1988).
[44] P. W. Berman, D. Dowbenko, C. C. Simonsen, and L. A. Laskey, *Science* **222**, 524 (1983).
[45] D. M. Kaetzel, J. K. Browne, F. Wondisford, T. M. Nett, A. R. Thomason, and J. H. Nilson, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 7280 (1985).

addition, the medium is supplemented with 10% (v/v) fetal calf serum, streptomycin sulfate (100 μg/ml), and penicillin (100 units/ml). The serum is heat-inactivated by treatment for 30 min at 56° in order to inactivate complement factors and potential virus and mycoplasma contaminants.

Cells are grown in 5% CO_2–air at 37°. Cultures are never incubated for more than 24 hr past the achievement of confluency, or else they become detached and/or sick.

The cells are typically subcultured at 1:20 twice per week. This is accomplished by detaching the cells from the plate by treatment with 0.05% (w/v) trypsin (1:250) (Gibco) in phosphate-buffered saline (PBS). Trypsinization is usually for 5 min at room temperature, but the exact time varies and the cells should be checked microscopically for rounding up. If overtrypsinized, the cells will clump. Monolayers are first rinsed with a few milliliters of trypsin solution before the final trypsin application. Trypsinization is stopped by removal of trypsin and cells are resuspended in serum-containing medium.

DHFR SELECTION CONDITIONS. Selection for DHFR-positive transformants is performed by subculturing cells 1:15 at 48 hr posttransfection into selective medium which lacks nucleosides. DHFR-selective medium may be either Ham's F12 minus thymidine and hypoxanthine or alpha medium minus nucleosides (obtained from Gibco). Either growth medium is supplemented with 10% dialyzed heat-inactivated fetal bovine serum and penicillin and streptomycin sulfate. The serum is dialyzed against PBS to remove low-molecular-weight nutrients such as nucleosides and amino acids which may act to bypass the selective conditions. Dialysis is performed in large dialysis 40-mm tubing (1 liter fetal bovine serum) versus 4 liters of PBS containing penicillin (10–20 unit/ml) and streptomycin (10–20 μg/ml) for 12–15 hr with one change of PBS during this time. The serum is then sterilized by filtration through a 0.2-μm nitrocellulose filter. Thus, the initial selection in medium minus nucleosides requires low levels of DHFR expression. Macroscopic colonies appear after 10 days and can be isolated after 14 days. If no colonies are observed the cells should be left to grow for another week. If DHFR is poorly expressed, cell growth is slower and colonies take longer to appear.

CONDITIONS FOR DHFR COAMPLIFICATION. The transformed DHFR-positive cells are expanded in DHFR-selective medium and subjected to increasing MTX selection. The initial selection for MTX resistance is accomplished by subculturing approximately 2×10^5 cells into a 10-cm tissue culture dish in DHFR-selective medium with either 0.005 or 0.02 μM MTX. The concentration is dependent on the efficiency of DHFR expression obtained from the transfected plasmid and should be determined experimentally. This MTX resistance selection should result in death of greater than 90% of the cells. At the appropriate concentration of

MTX, between 10 and several hundred colonies may appear within 2 weeks of selection. Since MTX-mediated cell death is slow (generally taking 3–5 days) and requires cell growth, it is important to ensure the cells under selection do not reach stationary phase of growth. If cells reach confluency within 4–5 days after growth in MTX selection, it is advised to subculture the cells 1:15 in the same concentration of MTX as well as to increase the concentration severalfold. The colonies which appear on individual dishes can be pooled and propagated for approximately 10 cell doublings in the same concentration of MTX. After the cells resume rapid growth, the concentration of MTX should be increased 2- to 5-fold and cells selected as described above. This process can be repeated until final levels of MTX resistance reach 1–10 μM MTX. Selection for resistance above this level does not usually yield significantly higher degrees of amplification. MTX (+) is obtained from Sigma (St Louis, MO).

DHFR Coamplification in Cells That Are Not DHFR-Deficient. Although DHFR is most frequently used as a recessive selectable marker in the DHFR-deficient CHO cells, several adaptations have made DHFR a useful dominant selectable and amplifiable genetic marker for cells that are not DHFR-deficient. These approaches rely on growth conditions that preferentially select for expression and amplification of the transfected DHFR gene over amplification of the endogenous DHFR gene. Although the appropriate concentration of MTX for selection varies widely between different cell lines, typical MTX concentrations for the initial selection step range from 0.1 to 0.5 μM.

COTRANSFECTION WITH ANOTHER DOMINANT SELECTABLE MARKER.[46] Cotransfection of both the gene of interest and the DHFR amplification marker with another dominant selectable marker [for example, pSV_2Neo encoding resistance to geneticin[47] (G418, Gibco)] can be used to isolate initial transformants. This selection for cotransformants yields a population of cells which have incorporated the heterologous DNA. This allows subsequent selection for amplification of the heterologous DNA, which usually occurs at frequencies above the frequency of amplification of endogenous DHFR genes. If one were to transfect and select directly for DHFR expression and amplification in a single step, the frequency of transfection (approximately 10^{-4}) would have to be multiplied by the frequency for amplification (around 10^{-3} to 10^{-4}) and would be less than the frequency of spontaneous amplification of the endogenous DHFR gene.

For this procedure, the transfected cells are first selected for resistance

[46] S. K. Kim and B. J. Wold, *Cell* **42**, 129 (1985).
[47] P. Southern and P. Berg, *J. Mol. Appl. Genet.* **1**, 327 (1982).

to the dominant selectable marker. In the case of pSV_2Neo, the colonies that appear after 2 weeks of selection for growth in 0.5–1 mg/ml G418 can be pooled and selected in moderate concentrations of MTX (usually around 0.3 μM) for amplification of the transfected DNA. Most frequently, the foreign DHFR gene is preferentially amplified over the endogenous DHFR gene.[46] For this approach, the vector pRK1-4 shown in Fig. 3 is a useful expression vector since it contains two transcription units: one which has a cloning site for insertion of a foreign coding region and which encodes a wild-type murine DHFR. After insertion of the desired coding sequences into this vector, the plasmid DNA can be cotransfected with a dominant selectable marker such as pSV_2Neo.

USE OF AN EFFICIENT DHFR EXPRESSION PLASMID. Another approach relies on use of a strong promoter for transcription of the foreign DHFR gene to permit sufficiently high levels of DHFR expression from the transfected DHFR gene to allow selection with MTX at concentrations above the natural resistance level of the cell.[20] Upon growth in the appropriate concentration of MTX, the transfected cells with amplified foreign DHFR genes can be selected over MTX-resistant cells which are a result of amplification of the endogenous DHFR gene.

USE OF MTX-RESISTANT DHFR EXPRESSION PLASMIDS.[48] Mutations within the DHFR coding region can render enzymes less sensitive to inhibition by MTX.[4] A murine DHFR cDNA which contains a single amino acid change from a leucine to an arginine at amino acid residue 22 encodes an altered enzyme which has a 270-fold elevated K_i relative to the wild-type enzyme.[4,49] It is possible to select directly for the presence of this enzyme by growth in moderate levels of MTX (0.5–1.0 μM).[49] In addition, the transfected DNA can be amplified by further selection in increasing concentrations of MTX. However, the large change in K_i makes it difficult to obtain high degrees of amplification with the mutant DHFR gene since lower levels of the enzyme are able to confer MTX resistance.

An alternative approach to MTX-resistant DHFR amplification vectors relies on the isolation of trimethoprim (a folic acid analog)-resistant DHFRs from microbial sources. In *E. coli* there are two plasmid-derived DHFRs that confer resistance to trimethoprim.[48] The type I DHFR is contained within the transposon Tn7 as part of the multidrug resistance episome R483. In contrast to the type I DHFR which is resistant to intermediate levels of trimethoprim, the type II enzyme is extremely resist-

[48] C. C. Simonsen, *in* "Molecular Genetics of Mammalian Cells" (G. M. Malacinski, ed.), p. 99. Macmillan, New York, 1986.

[49] C. C. Simonsen and A. D. Levinson, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 2495 (1983).

ant to trimethoprim. The genes for the type I[50] and the type II[51] enzymes can function as dominant MTX resistance selection markers in mammalian cells. However, since these DHFRs are extremely resistant to MTX, their amplification is not possible by MTX selection.

Monitoring Stability of Amplified DHFR Genes. The most direct method to monitor stability of the amplified genes is to remove the selective agent from the growth medium and monitor the gene copy number by Southern blot hybridization analysis as the cells propagate in the absence of selection. However, this is somewhat cumbersome and time consuming. The stability may be predicted by karyotypic analysis of the resistant cells. The presence of double minute chromosomes, tetraploidy, or dicentric or extremely aberrant chromosomes is usually indicative of instability.[16,18,21,23]

Alternatively, the stability of amplified DHFR genes can be predicted by analysis of the histogram of the DHFR content per cell for the population. This is performed by staining cells with a fluorescent analog of MTX (MTX-F) (from Molecular Probes, Eugene, OR) and analysis using a fluorescence-activated cell sorter.[52] MTX-F quantitatively stains cells for DHFR. The pattern of the histogram of fluorescence/cell can be used to predict stability. By this procedure, there is negligible staining of DHFR-deficient cells (Fig. 2a). An unstable MTX-resistant population exhibits a heterogeneous skewed distribution with many cells exhibiting low levels of DHFR (Fig. 2b). A stable MTX-resistant population exhibits a uniform population of highly fluorescent cells (Fig. 2c). For MTX-F staining, the cells are propagated for 2–3 days in the absence of MTX since MTX will effectively compete with MTX-F for DHFR binding. Then the staining of cells for DHFR is performed by addition of 10–30 μM MTX-F to the culture medium and incubation at 37° for at least 12 hr prior to analysis. After incubation, the cells are rinsed several times with medium, harvested by trypsinization, centrifuged at low speed, and resuspended in tissue culture medium for analysis of fluorescence intensity with a fluorescence-activated cell sorter.

Carbamoyl-Phosphate Synthase–Aspartate Transcarbamoylase–Dihydroorotase

Carbamoyl-phosphate synthase–aspartate transcarbamoylase–dihydroarotase (CAD) is a multifunctional protein that catalyzes the first three steps in UMP biosynthesis. The complex includes carbamoyl-phosphate

[50] C. C. Simonsen, E. Y. Chen, and A. D. Levinson, *J. Bacteriol.* **155,** 1001 (1983).
[51] K. O'Hare, C. Benoist, and R. Breathnach, *Proc. Natl. Acad. Sci. U.S.A.* **78,** 1527 (1981).
[52] R. J. Kaufman, J. R. Bertino, and R. T. Schimke, *J. Biol. Chem.* **253,** 5852 (1978).

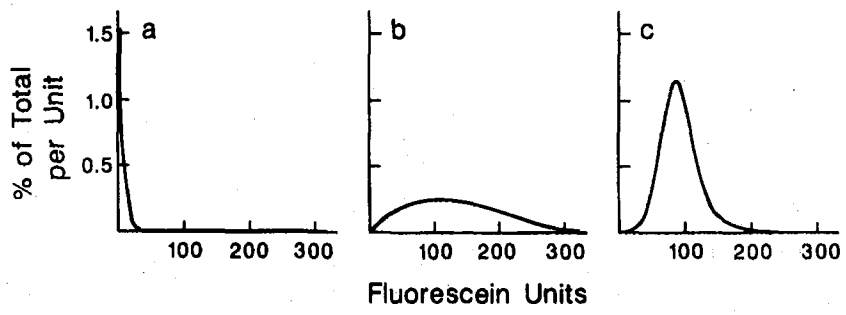

FIG. 2. MTX-F fluorescence of stable and unstable MTX-resistant populations. The distribution of DHFR content per cell determined by MTX-F fluorescence intensity per cell is shown for the original CHO DHFR-deficient cells (a), for a MTX-resistant cell line with unstable amplified DHFR genes (b), and for a MTX-resistant cell line with stable amplified DHFR genes (c).

synthase, aspartate transcarbamoylase, and dihydroorotase. PALA (*N*-phosphonoacetyl-L-aspartic acid) is a transition state analog inhibitor of the aspartate transcarbamoylase which can kill cells as a result of UMP depletion. Growth in sequentially increasing concentrations of PALA selects for amplification of the CAD gene.[53] In practice, the use of this selection system has been limited to CAD-deficient CHO cells.[54] The full-length cDNA for the mammalian gene has not been isolated intact, due to its large size. A cloned copy of the genomic CAD gene has been introduced into CAD-deficient CHO mutants (Urd-A)[55] by selection for growth in the absence of uridine as described below.[56] Individual transformants selected for growth in increasing concentrations of PALA exhibit amplification of the introduced CAD gene.[54] Alternatively, the *E. coli* aspartate transcarbamoylase gene *(PyrB)* carried in a eukaryotic expression plasmid can be selected and amplified up to 4-fold by PALA selection in aspartate transcarbamoylase-deficient CHO cells[57] as described below.

Conditions for Selection and Coamplification with CAD. A vector encoding the *E. coli pyrB* gene product (the catalytic subunit of aspartate transcarbamoylase) and the Tn5 phosphotransferase (geneticin, G418, resistance marker) in plasmid pSPN[57] can be used to transfer and amplify in CHO cells deficient in CAD (*Urd⁻A,* clone D20 from David Patterson, University of Colorado). *Urd⁻A* cells are maintained in Ham's F12 me-

[53] T. D. Kemp, E. A. Swryrd, M. Bruist, and G. R. Stark, *Cell* **9**, 541 (1976).
[54] G. M. Wahl, B. R. de Saint Vincent, and M. L. DeRose, *Nature (London)* **307**, 516 (1984).
[55] D. Patterson and D. V. Carnright, *Somatic Cell Genet.* **3**, 483 (1977).
[56] B. R. de Saint Vincent, S. Delbruck, W. Eckhaert, J. Meinkoth, L. Vitto, and G. Wahl, *Cell* **27**, 267 (1981).
[57] J. C. Ruiz and G. M. Wahl, *Mol. Cell. Biol.* **6**, 3050 (1986).

dium containing 10% (v/v) fetal calf serum and supplemented with 60 μM uridine.[58] At 48 hr posttransfection, cells are selected for CAD expression by plating 10^5 cells per 10-cm tissue culture dish in medium lacking uridine and containing 10% dialyzed fetal calf serum. Selection for amplification is performed by selection cells in 100 μM PALA (obtained from the National Institutes of Health) and then increasing the concentration to 250 μM and 1 mM as the cells become sequentially resistant to each concentration. It should be possible to select and amplify this plasmid in cells that are not CAD-deficient by initial selection for transformants that are G418 resistant and then increasing the selection for CAD by growth in uridine-free medium with increasing concentrations of PALA.

Adenosine Deaminase

Adenosine deaminase (ADA) is a useful dominant selectable and highly amplifiable genetic marker for mammalian cells.[59] Although ADA is present in virtually all mammalian cells, it is not an essential enzyme for cell growth. However, under certain conditions, cells require ADA.[60-63] Since ADA catalyzes the irreversible conversion of cytotoxic adenine nucleosides to their respective nontoxic inosine analogs, cells propagated in the presence of cytotoxic concentrations of adenosine or cytotoxic adenosine analogs such as 9-β-D-xylofuranosyl adenine (Xyl-A) require ADA to detoxify the cytotoxic agent for survival. Once functional, ADA is required for cell growth, then 2′-deoxycoformycin (dCF), a tight-binding transition-state analog inhibitor of ADA can be used to select for amplification of the ADA gene.[59] As a result of one selection protocol, ADA was overproduced 11,400-fold and represented 75% of the soluble protein synthesis of the cell.[64] ADA can function as a dominant selectable and amplifiable genetic marker[59] because most cells synthesize minute quantities of ADA. Thus, it is possible to select for introduction of a heterologous ADA gene carried in an efficient expression vector. One vector, pMT3SV$_2$ADA, useful for ADA coamplification is shown in Fig. 3. It contains a transcription unit to direct

[58] J. N. Davidson, D. V. Carnright, and D. Patterson, *Somatic Cell Genet.* **5,** 175 (1979).
[59] R. J. Kaufman, P. Murtha, D. E. Ingolia, C.-Y. Yeung, and R. E. Kellems, *Proc. Natl. Acad. Sci. U.S.A.* **83,** 3136 (1986).
[60] C.-Y. Yeung, D. E. Ingolia, C. Bobonis, B. S. Dunbar, M. E. Riser, J. J. Siciliano, and R. E. Kellems, *J. Biol. Chem.* **258,** 8338 (1983).
[61] G.-Y. Yeung, M. E. Riser, R. E. Kellems, and M. J. Siciliano, *J. Biol. Chem.* **258,** 8330 (1983).
[62] C. Fernandez-Mejia, M. Debatisse, and G. Buttin, *J. Cell. Physiol.* **120,** 321 (1984).
[63] P. A. Hoffee, S. W. Hunt III, and J. Chiang, *Somatic Cell Genet.* **8,** 465 (1982).
[64] D. E. Ingolia, C.-Y. Yeung, I. F. Orengo, M. L. Harrison, E. G. Frayne, F. B. Rudolph, and R. E. Kellems, *J. Biol. Chem.* **260,** 13261 (1985).

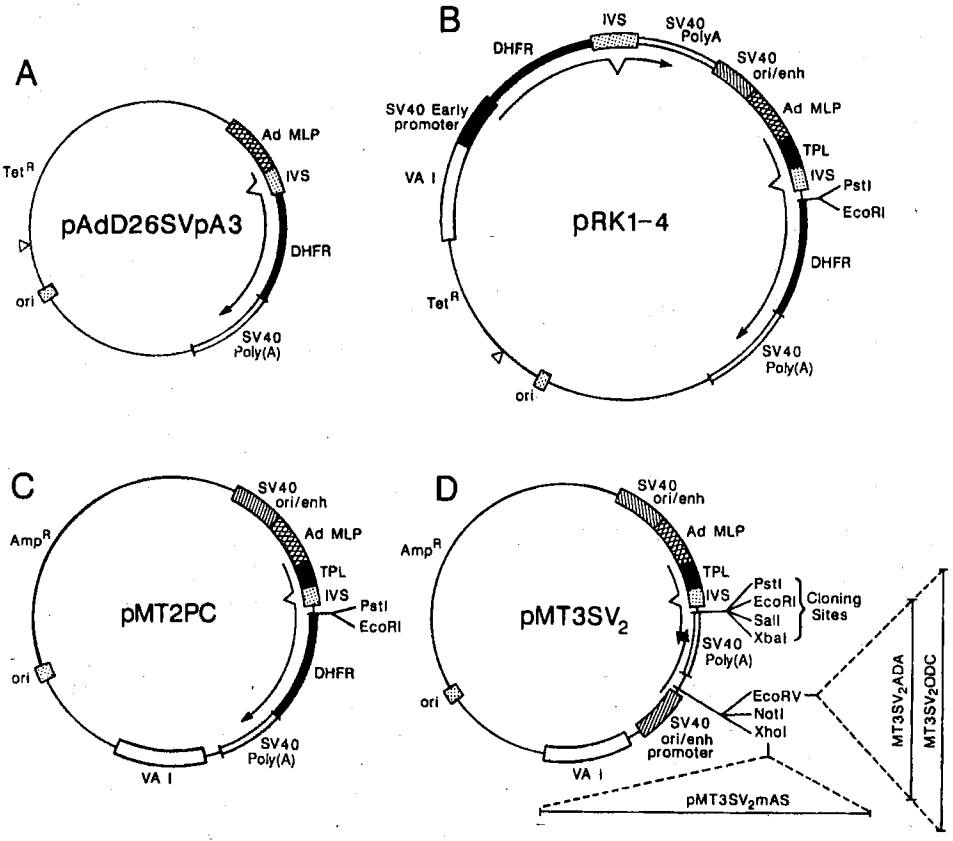

FIG. 3. Vectors for coamplification of heterologous genes. The components of the vectors described are as follows: *Tet^R* and *Amp^R*, tetracycline and ampicillin resistance genes for selection in *E. coli;* ori, bacterial origin of replication from pBR322 (A, B) or from pUC18 (C, D) for replication in *E. coli;* Ad MLP, adenovirus major late promoter for transcription initiation; IVS, intervening sequence composed of 5′ splice site from the adenovirus first leader of late mRNA and a 3′ splice site from an immunoglobulin gene for mRNA processing; TPL, a cDNA copy of the majority (2-$\frac{1}{3}$) of the adenovirus tripartite leader for efficient translation; SV40 ori/enh, origin of replication and enhancer element from SV40 for replication in COS monkey cells and for transcriptional activation; SV40 promoter, the early promoter of SV40 for transcription initiation; SV40 poly(A), polyadenylation signal from SV40 for transcription termination and mRNA processing; VA I, adenovirus VA I gene product to enhance translation. Selection markers: DHFR, dihydrofolate reductase; mAS, human asparagine synthetase; ADA, human adenosine deaminase; ODC, ornithine decarboxylase. The unique sites for cloning are indicated. (A) Enhancerless DHFR expression vector for cotransfection and coamplification. (B) DHFR selection vector with cloning sites for insertion for foreign coding region (*PstI*, *Eco*RI) into an efficient transcription unit. (C) Selection vector for expression of DHFR from a polycistronic transcript. (D) General vector with alternate amplifiable selection markers and several restriction sites for insertion of a foreign coding region into an efficient transcription unit.

expression of a coding region inserted into a unique cloning site and a separate transcription unit for ADA expression.

Conditions for Selection and Coamplification with ADA. Two selection schemes have been described which can be used for selection and amplification of ADA as a dominant selectable genetic marker.

XYL-A. The adenosine analog Xyl-A is cytotoxic due to its incorporation into DNA via conversion to the purine nucleotide catalyzed by adenosine kinase. Cells may become resistant to Xyl-A by action of ADA to inactivate the adenosine analog or by deletion of adenosine kinase. A selection scheme that allows for convenient transfer of ADA genes into a variety of cells involves the use of low concentrations of Xyl-A (4 μM) in the presence of 2'dCF (0.01–0.03 μM) to inhibit endogenous ADA. This Xyl-A selection is very cytotoxic and cells usually die within 48 hr. The Xyl-A selection is the most convenient scheme for introduction of heterologous ADA genes into a variety of cells. However, amplification by selection for increased resistance to higher concentrations of dCF is variable in different cell lines. The variability is most likely due to the frequency of deletion of adenosine kinase.[59,60] For this reason, the 11-AAU selection procedure was devised to allow for amplification of heterologous genes since growth in 11-AAU requires functional adenosine kinase.

11-AAU. The 11-AAU (adenosine, alanosine, uridine) selection protocol was modified from the selection protocol for adenosine kinase[65] by increasing the adenosine concentration 11-fold to 1.1 mM. In the presence of 1.1 mM adenosine, ADA is required to alleviate adenosine toxicity. In this selection, alanosine (50 μM) blocks *de novo* AMP synthesis and thus cells require adenosine kinase to convert adenosine to AMP. Since adenosine depletes phosphoribosylpyrophosphate (PRPP), resulting in the inhibition of endogenous pyrimidine synthesis, the medium is supplemented with uridine (240 μg/ml) to allow RNA synthesis to occur.[66]

For selection, 2 days after transfection, cells are subcultured 1:15 into medium containing 11-AAU with 0.03 or 0.1 μM dCF. Cells from one plate can be subcultured 1:15 into 11-AAU medium, with some plates containing 0.03 μM and the others containing 0.1 μM dCF. Cell death upon growth in the 11-AAU selection is slower than with the Xyl-A selection. Colonies appear at 10–14 days and are then pooled and grown in sequentially 3-fold increasing concentrations of dCF.

The results of selection for ADA are variable since different cell lines have differing capacities to grow in 11-AAU medium, in the absence of dCF. If required, it may be necessary to select first for a variant of the host

[65] T. S. Chan, R. P. Creagan, and M. P. Reardon, *Somatic Cell Genet.* **4**, 1 (1978).
[66] H. Green and T. Chan, *Science* **182**, 836 (1973).

cell line that is capable of growing in 11-AAU. The basis of this initial selection for growth in 11-AAU is unknown. For selection in CHO cells, it is not necessary to preselect for growth in 11-AAU. Once a cell line is obtained that can grow in 11-AAU, then heterologous ADA genes can be transfected and selected for by growth in 11-AAU with addition of dCF (0.03–0.1 μM).

Since fetal calf serum contains significant, variable levels of bovine ADA, it is important to prepare selection medium fresh prior to use. For convenience, the selection medium may be made and stored without serum. Ten percent serum can be added just prior to use. For appropriate selection, it is advisable to feed the cells frequently (every 4–5 days) with fresh selection medium. This is especially true when using the Xyl-A selection procedure. As an alternative to fetal calf serum, horse serum, which has significantly lower levels of ADA, should be used if it can support the growth of the host cells. Xyl-A and alanosine can be obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute. 2′-Deoxycoformycin can be obtained from Sigma (St. Louis, MO).

Multidrug Resistance

Cross-resistance to a variety of lipophilic cytotoxic agents such as adriamycin, colchicine, vincristine, and actinomycin D frequently results from overproduction of a 170-kDa plasma membrane glycoprotein (P-glycoprotein) due to gene amplification.[67] This membrane protein is involved in transport of these drugs out of the cell.[68–70] Some multiple drug-resistant (mdr) cell lines are significantly more resistant to the drug used in their selection than to other drugs.[71] The preferential resistance found for colchicine transport can be accounted for by the appearance of mutations within the P-glycoprotein structural gene that preferentially confer resistance to colchicine.[72] Thus, expression of different cDNA clones derived from cells resistant to different drugs may confer preferential resistance to the drug for which the original cells were initially selected. The cloned murine mdr and human mdr1 cDNAs can function as dominant selectable

[67] J. R. Riordan, K. Deuchars, N. Kartner, N. Alon, J. Trent, and V. Ling, *Nature (London)* **316**, 817 (1985).
[68] G. F.-L. Ames, *Cell* **47**, 323 (1986).
[69] P. Gros, J. Croop, and D. Housman, *Cell* **47**, 371 (1986).
[70] G.-J. Chen, J. E. Chin, K. Ueda, D. P. Clark, I. Pastan, M. M. Gottesman, and I. B. Roninson, *Cell* **47**, 381 (1986).
[71] K. W. Scott, J. L. Biedler, and P. W. Melera, *Science* **232**, 751 (1986).
[72] K. Choi, C. Chen, M. Driegler, and I. B. Roninson, *Cell* **53**, 519 (1988).

and amplifiable genetic markers.[73,74] The potential degree of gene amplification using mdr may be limited since cells exhibiting very high levels of drug resistance contain only 50 copies of the gp170 gene.[73] The human mdr1 gene has been used to coamplify major excreted protein (MEP) in NIH 3T3 cells.[74] In this case, there was approximately 10-fold amplification as colchicine resistance increased from 80 ng/ml to 1 μg/ml of colchicine.

Conditions for Selection and Coamplification with mdr. At 48 hr posttransfection, cells are subcultured at $2-4 \times 10^5$ cells/10-cm dish in growth medium containing 60 ng/ml of colchicine. Clones or pools of transformants are then grown in 80 ng/ml colchicine and are selected in 2-fold increments of colchicine. At each step, the cells should grow for 5–10 days before being plated into the next higher step. The particular concentration of colchicine used may vary depending on the expression vector and the particular cDNA encoding mdr. It has been possible to select for mdr transformants with as little as 7 ng/ml colchicine.[74]

An alternate to colchicine resistance selection for mdr is to select for adriamycin resistance. At 0.1 μg/ml of adriamycin, the murine mdr cDNA carried in an efficient expression vector was capable of transmitting resistance to transfected cells.[73]

Ornithine Decarboxylase

Ornithine decarboxylase (ODC) is the first enzyme in the synthesis of polyamines and is an essential enzyme for cell growth.[75] An expression plasmid encoding the ODC cDNA can complement the growth of ODC-deficient CHO cells[76] in medium lacking putrescine.[77] In these cells, it is possible to select for greater than 700-fold amplification of the ODC gene by growth in increasing concentrations of the suicide-substrate inhibitor difluoromethylornithine (DFMO). By introduction of the ODC cDNA into an efficient expression vector it is possible to select directly for ODC in wild-type cells by growth in moderate concentrations of DFMO.[77] Selection in increasing concentrations of DFMO has resulted in a 300-fold amplification of the transfected gene in wild-type cells.

Conditions for Selection and Coamplification of ODC. At 48 hr posttransfection, cells are subcultured at 10^5/10-cm dish into medium contain-

[73] P. Gros, Y. B. Neriah, J. M. Croop, and D. E. Housman, *Nature (London)* **323**, 728 (1986).
[74] S. E. Krane, B. R. Troen, S. Gal, K. Veda, I. Pastan, and M. M. Gottesman, *Mol. Cell. Biol.* **8**, 3316 (1988).
[75] C. W. Tabor and H. Tabor, *Annu. Rev. Biochem.* **53**, 749 (1984).
[76] C. Steglich, A. Grens, and I. E. Scheffler, *Somatic Cell. Mol. Genet.* **11**, 11 (1985).
[77] T.-R. Chiang and L. McConlogue, *Mol. Cell. Biol.* **8**, 764 (1988).

ing DFMO at 160 μM. The plates are refed every 5 days until a resistant population emerges. Multiple colonies are pooled and selected sequentially for resistance to 600 μM, 1 mM, 3 mM, 9 mM, and 15 mM DFMO. DFMO may be obtained from Merrell Dow Research Institute (Cincinnati, OH).

Asparagine Synthetase

Asparagine synthetase (AS) is a housekeeping enzyme responsible for the biosynthesis of asparagine from glutamine and aspartic acid. Selection for expression of heterologous AS in cells is possible by selection for growth in asparagine-free medium with addition of an appropriate concentration of an inhibitor of AS. Two AS inhibitors which can be used are albizziin, which is a glutamine analog, and β-aspartyl hydroxamate (β-AHA), an aspartic acid analog.[78] The use of AS as a dominant selection and amplification marker relies on its efficient expression to select its amplification over that of the endogenous AS gene.

The *E. coli* AS gene is more convenient to use as a dominant selectable and amplifiable marker. Since the *E. coli* enzyme uses ammonia instead of glutamine as a nitrogen source, it is resistant to albizziin. Mammalian AS uses glutamine as a nitrogen source and so is inhibited by albizziin. Dominant selection for the bacterial AS gene is possible by growth in asparagine-free medium containing albizziin to inhibit the endogenous mammalian AS. Subsequently, the transfected DNA can be amplified up to 100-fold by growth in increasing concentrations of β-AHA.[79]

Cotransformation and Coamplification with Bacterial AS. For cotransfection and coamplification with AS, plasmid DNA encoding the desired gene product is cotransfected with the bacterial AS expression plasmid pSV_2-AS[79] [pSV_2-AS can be obtained from Clifford Stanners (McGill University, Quebec, Canada)]. Forty-eight hours after transfection, cells are rinsed with selective medium and subcultured at 2×10^5/10-cm dish in selective medium. Selective medium consists of α-MEM lacking asparagine, with 30 μg/ml of glutamine, 10% dialyzed fetal calf serum, and 2 mM albizziin (purchased from Sigma). The exact concentration of albizziin required for death of untransfected cells should be determined for each cell type.

For amplification, transfected clones are grown in asparagine-free medium with 30 μg/ml of glutamine and containing 0.1 mM β-AHA (β-AHA can be obtained from Sigma). Resistant cells are cultured in medium containing increasing concentrations of β-AHA starting at 0.2 mM and

[78] I. Andrulis, J. Chen, and P. Ray, *Mol. Cell. Biol.* 7, 2435 (1987).
[79] M. Cartier, M. Chang, and C. Stanners, *Mol. Cell. Biol.* 7, 1623 (1987).

increasing in successive steps of 0.2 m*M* up to 1.5 m*M*. From this concentration up to 5 m*M* β-AHA, the steps are 1 m*M* each.

Cotransformation and Coamplification with Mammalian AS. Generally better success for amplification is obtained by using a mammalian AS expression vector and albizziin as the selection agent. One vector, pMT3SV$_2$mAS (Fig. 3D), contains the human AS gene[78] under transcriptional control of the SV40 early promoter. Selection for this vector in transfected cells is performed by growth in asparagine-free medium containing 2 m*M* glutamine and 2 m*M* albizziin. Resistant transformants can be amplified by increasing albizziin stepwise up to 50 m*M*. Further selection can be accomplished by reducing 10-fold both the concentration of glutamine (to 0.2 m*M*) and of albizziin. Then selection for further increases in albizziin resistance can be performed.

Other Strategies for Amplification of Transfected DNA

In addition to the drug selections described above, transformants can be selected on the basis of expression of surface antigens [for example, the T cell antigen leu-2[80] and the transferrin receptor[81]] by cell sorting using a fluorescence-activated cell sorter and specific antibodies. Sequential selection for positive fluorescence yields cells that have amplified the gene for the surface antigen.[82] Other fluorescently labeled compounds that can be used to select cells include a fluorescent conjugate of methotrexate to select for elevated DHFR levels,[52] or aryl hydrocarbons to select for cells exhibiting elevated levels of aryl hydrocarbon hydroxylase.[83] It is also possible to isolate living cells by staining of living cells for β-galactosidase activity that is introduced by transfer of specific chimeric genes encoding β-galactosidase.[84]

Other Dominant Selectable Markers

The most commonly used dominant selectable marker confers resistance to neomycin, kanamycin, and similar compounds in bacterial cells. It is encoded by the bacterial transposon Tn5 aminoglycoside phosphotransferase gene *(Neo^r)*. G418 (obtained from Gibco) is a compound related to neomycin, which can act in mammalian cells to block translation. The *Neo^r* gene introduced into a mammalian expression vector (pSV$_2$neo)[47]

[80] P. Kavathas and L. A. Herzenberg, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 524 (1983).
[81] A. McClelland, L. C. Kuhn, and F. H. Ruddle, *Cell* **39**, 267 (1984).
[82] P. Kavathas and L. A. Herzenberg, *Nature (London)* **306**, 385 (1983).
[83] A. G. Miller and J. P. Whitlock, Jr., *Mol. Cell. Biol.* **2**, 625 (1982).
[84] G. P. Nolan, S. Fiering, J. F. Nicholas, and L. A. Herzenberg, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 2603 (1988).

can confer G418 resistance to mammalian cells. For selection, cells are grown in 0.5–1.0 mg/ml G418. Similarly, the hygromycin B phosphotransferase gene from *E. coli,* when introduced into mammalian cells in an appropriate expression vector (pHyg 141-31, obtained from B. Sugden, University of Wisconsin, Madison, WI), can confer resistance to the antibiotic hygromycin.[85] Hygromycin can be obtained from Calbiochem (San Diego, CA) and is generally used at 100–300 μg/ml. The biological half-life of hygromycin in solution at 4° is approximately 10 days. G418 and hygromycin resistance selection schemes are independent, and both resistance genes can be selected for in cells transfected with both genes. However, neither of these selection markers has been demonstrated to be amplifiable.

Strategic Considerations

In designing an approach to obtain efficient expression of a heterologous gene through coamplification, several considerations should be taken into account: (1) the method of DNA transfer, (2) the vector for coamplification, and (3) the protocol for selection and cloning of the cells.

Methods of DNA Transfer

Since a detailed description of the different methods for transfer of DNA into mammalian cells is beyond the scope of this article,[37] discussion will be limited to how the different methods may influence the selection protocol for gene amplification. The most common method for gene transfer is to add DNA directly to the cells in the form of a $CaPO_4$ coprecipitate. Transfection frequencies may vary widely between cell lines to as high as 1 transformant per 10^4 transfected cells.[33] Via this approach DNA enters the cell though an endocytic vesicle. As a consequence, the DNA frequently becomes rearranged, possibly as a result of passage through cellular compartments of low pH or containing endonucleases. In contrast, other methods directly introduce DNA into the cytoplasm of the cell. One of these approaches relies on the polyethylene glycol (PEG)-induced fusion of bacterial protoplasts with mammalian cells.[86] This method can be very efficient (10^{-2}–10^{-4}/cell) and can be used for cells that are difficult to transfect by the $CaPO_4$ procedure. Protoplast fusion frequently yields multiple copies of the plasmid DNA tandemly integrated into the host chromosome in a head-to-tail array.[56] Since it is not likely that two different plasmids harbored in two different bacteria will integrate into the same cell, protoplast fusion is not useful for cotransfection of independent

[85] J. L. Yates, N. Warren, and B. Sugden, *Nature (London)* **313**, 812 (1985).
[86] W. Schaffner, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 2163 (1980).

plasmids, in contrast to $CaPO_4$ cotransfection. Thus, protoplast fusion necessitates that the selection and amplification marker be on the same plasmid as the gene of interest. Similarly to protoplast fusion, electroporation can efficiently introduce DNA directly into the cytoplasm of recipient cells.[87,88] Electroporation does not require the selection gene and gene of interest to be on the same plasmid. However, there is less experience with this method to compare it directly with protoplast fusion and $CaPO_4$-mediated cotransfection for propensity to generate rearrangements.

Selection Vectors for Coamplification

There are two classes of vectors for coamplification: (1) those in which the product gene transcription unit is on a separate plasmid than the selection gene transcription unit, and (2) those in which the product and selection gene transcription units are contained within the same vector. The efficiency of cotransfection of separate plasmids depends on the ability of the two DNAs to be ligated within the cell. It is also possible to linearize and ligate the selection and product transcription unit plasmids before transfection. One advantage with using separate plasmids is that the ratio of the product and selection gene can be varied in favor of the product gene to ensure a greater copy number relative to the selection gene copy number. One approach to select for the cells which have incorporated both the selection gene and the product gene is to use a defective expression vector for the selection gene, for example, one that has deleted an enhancer element. In this case, cotransfection with the product gene plasmid containing an enhancer element yields cells which efficiently express the selection gene only when its transcription unit has become linked to the product gene. By this approach, the enhancer-deficient DHFR expression plasmid pAdD26SVpA3[38] (Fig. 3A) is useful for cotransformation of CHO DHFR-deficient cells and coamplification by MTX resistance selection.

Two types of vectors that have both the selection gene and the product gene within the same plasmid have been described. The first contains one transcription unit encoding the selection marker and another transcription unit containing a unique cloning site for insertion of heterologous coding regions. Different vectors described (Fig. 3B,D) have been constructed to contain DHFR, ADA, mdr, and AS. These vectors are useful for protoplast fusion and ensure that cells transformed with the selection marker will likely also incorporate the product gene.

Another approach relies on the ability of ribosomes to translate internal

[87] H. Potter, L. Weir, and P. Leder, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 7161 (1984).
[88] K. Shigekawa and W. J. Dower, *BioTechniques* **6**, 742 (1988).

cistrons within polycistronic mRNAs. The "scanning" hypothesis for translation initiation states that ribosomes bind to the 5′ end of capped mRNAs and migrate in the 3′ direction until they encounter an AUG in an appropriate context that can serve as an initiator codon.[89] However, there is a low level of internal initiation of translation at internal AUG codons. This inefficient translation at internal AUG codons may yield sufficient quantities of gene product to allow selection for transcription units which harbor selection genes within the 3′ end of the transcript.[90] The vector pMT2PC (Fig. 3C) contains a DHFR coding sequence within its 3′ end which can be used for DHFR selection in CHO DHFR-deficient cells. Since one proposed mechanism of this internal initiation involves translation termination, continued scanning, and reinitiation, it is important that no AUG codons are present between the termination codon for the 5′ open reading frame and the DHFR coding region. The optimal number of bases between the two coding regions is estimated to be approximately 100 in order to optimize DHFR translation.[91] Cell lines which are selected for heterologous gene expression by DHFR expression by this approach generally express 10-fold greater levels of the heterologous gene which is inserted into the cloning site at low levels of MTX selection. However, at high levels of MTX resistance selection, the 5′ open reading frame frequently becomes deleted to allow more efficient DHFR expression. Thus, while higher expression levels may be obtained earlier in selection, the maximal expression obtained with vectors encoding polycistronic transcription units is not effectively greater than that obtained by other approaches.

Protocols for Selection for Coamplification

Two strategies for transformant selection and coamplification are as follows. In scheme A (Fig. 4), individual clonal tranformants are isolated and subsequently independently grown in increasing concentrations of the selection agent for gene amplification. In scheme B (Fig. 4), a pool of transformants is collected and grown in increasing concentrations of the selection agent for gene amplification. The final resistant pool is cloned by limited dilution plating at the end of the selection process. The advantage of scheme B is that it requires less effort to amplify larger numbers of individual transformants within the pool. Cells selected are those that become resistant to increasing MTX because the heterologous DNA has integrated into a chromosomal position that is efficiently expressed and amplified. Approximately 30% of the selected pools exhibit coamplifica-

[89] M. Kozak, *Cell* **22**, 7 (1980).
[90] R. J. Kaufman, P. Murtha, and M. V. Davies, *EMBO J.* **6**, 187 (1987).
[91] M. Kozak, *Mol. Cell. Biol.* **7**, 3438 (1987).

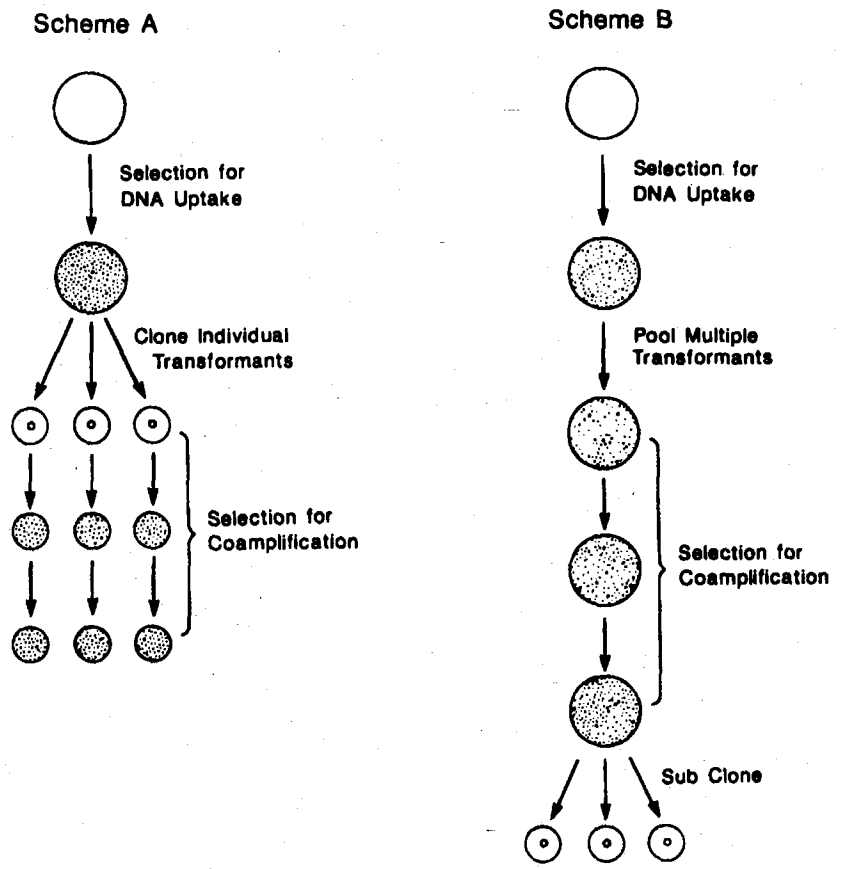

FIG. 4. Strategies for selection for amplification of heterologous genes.

tion of the desired gene with the selection gene. After several rounds of increasing MTX selection, the final cell line obtained is usually composed of cells derived from one or a very few transformants. In contrast, when high-producing individual clonal transformants are selected for amplification as depicted in scheme A (Fig. 4), it is infrequent that the cotransfected gene is coamplified with the selection gene.[38] One disadvantage of scheme B (Fig. 4) is the potential to lose good producing cell lines. It is possible to avoid this by cloning after the first step in the selection for coamplification and subsequently selecting the cloned lines for increased coamplification. The success of the particular strategy depends on the type of expression vector used and the method of DNA transfer. For this reason, the selection protocols provided below are examples that have proved most successful for a particular vector system and mode of DNA transfer.

Cotransformation by $CaPO_4$ Cotransfection with DHFR

1. DNA from an enhancer containing vector encoding the product gene (25 μg) is coprecipitated with pAdD26SVpA3 (2.5 μg) in 0.3 *M* sodium acetate, pH 4.5 (300 μl) and 2.5 volumes of ethanol at −20° for 20 min.

2. DNA is pelleted by centrifugation for 5 min in an Eppendorf centrifuge at 4°. The supernatant is removed. In the tissue culture hood (the operator must wear gloves), the tubes are decanted and turned over to drain. The DNA pellets are air dried in the hood. The tubes should have no residual ethanol before continuing.

3. DNA is resuspended in 2 × HEBSS (0.25 ml) (2 × HEBSS: HEPES, 10 g/liter; NaCl, 16 g/liter; KCl, 0.74 g/liter; $Na_2HPO_4 \cdot H_2O$, 0.25 g/liter; dextrose, 2 g/liter; pH 7.05). The appropriate pH of the HEBSS is crucial and varies depending on the cell line. Typically, buffers of varying pH should be prepared and tested for each cell line for optimal transfection efficiency. The 2 × HBSS should be stored at −70°. The solution may be vortexed lightly to ensure the DNA is in solution.

4. To the 2 × HEBSS–DNA suspension, an equal volume of 2 × $CaCl_2$ (2 × $CaCl_2$, 0.25 *M*) is added slowly while bubbling. The 0.25 *M* $CaCl_2$ can be stored at −20°. The $CaPO_4$–DNA precipitate is briefly vortexed and allowed to rest for 20 min at room temperature. It is important that the precipitate be very fine and it should appear opalescent.

5. Medium is removed from CHO DHFR-deficient cells which were subcultured at 5×10^5/10-cm dish 24 hr prior to transfection. The precipitate is then applied and the cells are incubated for 30 min at room temperature.

6. Then 4.5 ml of alpha medium with 10% fetal calf serum is added without removing the DNA precipitate and the cells are incubated at 37° for 4–5 hr.

7. The medium is then removed and 2 ml of alpha medium containing 10% glycerol is added for 3 min at room temperature.

8. The glycerol is removed and cells rinsed and fed with alpha medium containing 10% fetal calf serum, and 10 μg/ml each of thymidine, adenosine, and deoxyadenosine. Penicillin (100 U/ml) and streptomycin (100 μg/ml) are also included.

9. Two days later, cells are subcultured 1 : 15 into alpha medium.

10. After 10–14 days, approximately 10–100 colonies should appear per dish. Pools of 10–100 colonies are made and are selected for resistance to increasing concentrations of MTX. Selection of 6 pools for amplification will most likely ensure success in coamplification of the heterologous product gene.

For a control for the transfection and selection, it is good to include cells that do not receive the DHFR expression plasmid. Under appropriate selection, no colonies should appear in cells that did not receive a DHFR plasmid.

Coamplification with Vectors Containing Transcription Units for Both Selection Gene and Product Gene. The foreign DNA coding region is inserted into a cloning site within the appropriate expression vector, as shown in Fig. 3B,D. The DNA can be introduced into cells by electroporation after linearization by restriction endonuclease digestion of naked DNA or by protoplast fusion. Below is a protocol for protoplast fusion.

PREPARATION OF PROTOPLASTS

1. *Escherichia coli* HB101 or DH5 harboring the expression plasmid and resistant to either ampicillin or tetracycline are used for protoplast fusion. A fresh overnight culture of bacteria is inoculated (100 μl) into 50 ml of L broth containing 8–10 μg/ml tetracycline or 50 μg/ml ampicillin and incubated at 37° on a rapid shaker for approximately 5–6 hr (depending on the strain), to an $A_{600\,nm}$ of 0.6. Bacterial growth should be carefully monitored during the growth period so the 0.6 A_{600nm} is not surpassed.

2. Chloramphenicol is added to 250 μg/ml (from a 125 mg/ml stock) and the culture incubated at 37° for an additional 12–16 hr in order to amplify the plasmid copy number. At the end of this incubation, the cultures can rest on ice for several hours if necessary until ready to continue. It is advisable at this point to isolate plasmid DNA from a small portion of the bacteria by a miniplasmid preparation procedure[92] and analyze it by restriction endonuclease digestion and gel electrophoresis. This analysis will ensure that no plasmid rearrangements have occurred during propagation of the cells to prepare protoplasts.

3. Centrifuge 50 ml at 3000 rpm for 10 min at 4°. From this point it is important to keep the bacteria on ice-water.

4. Resuspend the pellet in 2.5 ml of chilled 20% sucrose in 50 m*M* Tris-HCl, pH 8.0.

5. Add 0.5 ml of a freshly prepared solution of lysozyme (5 mg/ml in 0.25 *M* Tris-HCl, pH 8.0), and swirl the tube very gently to mix.

6. Incubate on ice for 5 min.

7. Add 1 ml of 0.25 *M* EDTA, pH 8.0.

8. *Slowly* add 1 ml of 0.05 M Tris-HCl, pH 8.0, one drop at a time.

9. Incubate at 37° in a water bath for 15 min.

10. Carefully dilute the protoplast solution with 20 ml of prewarmed

[92] T. Maniatis, E. F. Fritsch, and J. Sambrook, "Molecular Cloning: A Laboratory Manual," Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1982.

DME medium containing 10% sucrose and 10 m*M* $MgCl_2$. Let the solution drip down the side of the tube and invert the tube slowly and gently to mix. It is important to be gentle at this point in order to not disrupt the protoplasts.

11. Hold at room temperature for 15 min (the protoplasts can remain in the hood for up to 2 hr, and at 4° for up to 2–3 days). Before doing the fusion, invert the tube several times, gently. At this time the solution will be slightly viscous and consists of protoplasts which are bacteria with their cell walls stripped. Removal of the cell wall now permits fusion of the cell membrane with that of the mammalian cell, an event that is potentiated by polyethylene glycol treatment.

PROTOPLAST FUSION

1. One day before fusion, subculture cells into 6-well plates (Costar Cluster 6 #3506; 24-mm diameter/well). Inoculate only 3 wells/plate at 1.5×10^5 cells/ml, 5 ml/well in complete medium. Incubate overnight until attached. It is better to seed the cells earlier in the day in order to give them maximum time for attachment.

2. Prepare polyethylene glycol (PEG) solution: Solid PEG (Baker, Phillipsburg, NJ; U218-9, average MW 950–1050) is melted by microwaving in a 50-ml conical test tube for 1–2 min until liquid. The PEG is then diluted with an equal volume of prewarmed Dulbecco's minimal essential medium (DMEM) (50/50, v/v) and is kept at room temperature.

3. Rinse the cells three times with serum-free medium.

4. Add 1.5 ml of protoplast suspension per well.

5. Centrifuge for 5 min at 1500 rpm (only two dishes at a time) in a table-top centrifuge with appropriate adapters (IEC Centra-7).

6. Aspirate by tilting the wells gently.

7. Add 1.5 ml of PEG/well and leave on for 1 min (for CHO cells). The PEG time will vary for different cell types. The appropriate time must be determined accordingly by testing for cell death and efficiency of transformation. The time may vary from 15 sec to 2 min.

8. The cells are rinsed six times with serum-free medium. Do not tip the plate when aspirating PEG from the wells. Dilute the wells with 5 ml of the medium and then aspirate it gently from one angle of the well, aspirating with one hand while adding medium in the well with the other hand.

9. Add 5 ml of complete medium containing 100 μg/ml kanamycin (Flow laboratories, McLean, VA; #16-720-48; 5000 μg/ml = 50×). This will kill any bacteria that have escaped conversion to protoplasts.

10. Incubate for 48–72 hr at 37°, then subculture cells into 5 × 10cm plates/well into selective medium.

11. After colonies appear, pools of 25–300 transformants/pool are prepared and selected for increasing resistance to selective agent for amplification.

Coamplification Using Polycistronic mRNA Expression Vectors. Polycistronic mRNAs can be translated in mammalian cells; however, the efficiency of translation of the internal cistron is variable and inefficient.[89-91] Insertion of a coding region upstream from DHFR within the expression vector pMT2PC (Fig. 3C) yields a bicistronic transcript in which DHFR is poorly translated. However, selection for the low level of DHFR expression from this vector is possible by growth selection of DHFR-deficient CHO cells in the absence of nucleosides. Since there is a strong selection for deletion of the upstream open reading frame when selecting for expression of the 3′ open reading frame in polycistronic vectors, it is important to take precautions to minimize the frequency of deletions as well as the potential for selection cells harboring deletions within the 5′ open reading frame to outgrow the population. For this reason, the DNA should be introduced by an efficient means and one which does not frequently result in rearrangements, such as protoplast fusion or electroporation. In addition, the strategy for transformant amplification should follow that of Fig. 4 scheme A, in which independent clonal transformants are isolated and subsequently grown in increasing selection for amplification. If one were to pool the transformants as in scheme B, the likelihood is greater that a cell harboring a deletion will outgrow. In addition, the increasing steps of selection for gene amplification should be small to encourage amplification and also to minimize deletion. Although this approach has potential difficulties, the advantages are that it may render higher degrees of expression at early stages of the amplification process.

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